

Phylogenetic Diversity of Organophosphorous Pesticide-Degrading Coral Bacteria from Mid-West Coast of Indonesia

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Abstract: The present study aimed to investigate the general insights into the diversity of the bacterial community associated with the corals which capable of degrading organophosphorous pesticide. The diversity of indigenous bacteria associated with corals from several sites in the Indonesia coastal waters able to degrade organophosphorous compounds (OPs) was investigated using of culture-based methods and molecular analyses. Twenty five strains among 103 isolates (24.36%) demonstrated their capability of degrading selected organophosphates (diazinon, chlorpyrifos, profenofos and ethion) as sole source of carbon and energy. A rapid grouping of the 25 selected isolates by using repetitive extragenic palindromic (rep)-PCR genomic fingerprinting with ERIC and BOXAIR primers was carried to estimate the richness of the isolates and 6 representative strains were examined further. Following partial sequencings of the 16S rDNA, it was shown that these strains belonged to three major groups of bacteria: (i) members of the division *Bacillus*, (ii) *Actinobacteria* and (iii) γ -Proteobacteria. Strain KM5, JM33, BM5, SB3, KF4 and BY6 were closely related to *Brachybacterium* sp., *Kytococcus* sp., *Brevibacterium* sp., *Chromohalobacter* sp., *Oceanobacillus* sp. and *Bacillus* sp., respectively. This study provides the first evidence of organophosphorous pesticide-degrading bacteria isolated from corals.

Key words: Coral, bacteria, degradation, organophosphorous, rep-PCR, 16S rDNA

INTRODUCTION

Coral reef is an ecosystem in the tropical ocean floor which has been built primarily by scleractinian corals and coralline algae, together with other commensal organisms including a variety of flatworms, polychaete worms, shrimps, crabs, brittle stars, molluscs and fish (Veron, 1986). About 85.707 km² or 14% of total corals in the world are extending all the way in the Indonesian sea (Tomascik *et al.*, 1997). Some benefits have been provided by corals to fulfill the human life necessities, such as, drugs, mariculture and nutrients, spawning, nursery, feeding ground and growing place for school of fish and as barrier of coastal erosion (Sammarco and Coll, 1992; Ben-Haim and Rosenberg, 2004). It has commonly been known that in developing countries, reefs near coastal areas, are under serious stress from coral mining, cyanide, blasting and land pollution in particular agricultural runoff as the results of the application of pesticides to control the pests and weeds.

In Indonesia, the use of herbicides began in the 1960's when the government launched plantation rehabilitation programme. Due to the undesirable effects on environmental quality, the usage of organochlorine

compounds was banned during the 1980s. Subsequently, organophosphates are being increasingly used to substitute for the organochlorines due to their rapid breakdown into environmentally safe products. However, most of these compounds have far more immediate toxicity than DDT and other related products (Wolterding, 1981). In most cases, when reefs are polluted and when the stress is severe or lasts long enough, the corals will die. Glynn *et al.* (1984) reported that herbicides killed corals at low concentration (0.02 mg L⁻¹) under brief exposure. In the previous study, Sabdono *et al.* (2007) reported that organophosphate compounds were detected in dead coral tissues.

It is well known that microorganisms attach firmly to almost any surface submerged in marine environments in which the cells grow, reproduce and produce extracellular polymers that provide structure to the assemblage termed as biofilm. Prokaryotes is believed as the most diverse component of coral reef communities (Rohwer *et al.*, 2002). In addition, bacteria are abundant and active around and in the coral surface layer (Kushmaro *et al.*, 1997). Furthermore, Kim (1994) mentioned that it is not surprised coral surfaces are often covered by microorganisms mostly are undestructive to corals.

Several studies on interactions among marine bacteria on the surface of corals have been reported by Kim (1994), Shashar *et al.* (1994), Ritchie and Smith, (1997), Kushmaro *et al.* (1997), Rohwer *et al.* (2001) and Friaiz-Lopez *et al.* (2002), however, it is expected that still quite a few parts of culturable coral-associated microorganisms are unexplored in the reef environments. Thus, such information might be desirable, as some of these bacteria may serve beneficial purposes in protecting corals from pesticides pollution by acting as film protection.

Characterization of soil pesticide-degrading bacteria have been extensively reported by Kelly *et al.* (1989), Haugland *et al.* (1990), Daugherty and Karel (1994) and Zipper *et al.* (1998). However, there is a serious lack of information on works directed toward pesticide-degrading marine microorganisms so far. In this context, it is of importance to assess the diversity of coral-associated microbial populations with specific consideration on the pesticide-degrading part, which has been up till now strongly neglected in comparison to soil microbial population.

In this study, we carried out an assessment on diversity of coral-associated bacteria capable of degrading organophosphorous pesticides from reef environment. To our knowledge, this is the first study on organophosphorous pesticide-degrading bacteria associated with coral, in particular from tropical reef environments.

MATERIALS AND METHODS

Sampling and isolation of coral-associated bacteria:

Corals representing 4 different life forms (branching, massive, sub-massive and foliose) were collected from Java, Bali, Sulawesi and Komodo coastal waters during August 2005 (Fig. 1). Specimens of the corals were collected randomly by scuba diving at depths of 2 to 3 m, broken away with chisel and hammer and placed in plastic bag submerged in sea water. Upon collection coral fragments were put into sterile plastic bags (Whirl-Pak, Nasco, USA), stored in cool box and immediately brought to our laboratory.

The corals were immediately rinsed with sterile seawater and scrapped with sterile knife. The resultant tissues were then tenfold serially diluted and the dilution was spread on ½ strength ZoBell 2216E marine agar medium incubated at room temperature for 48 h. On the basis of morphological features, colonies were randomly picked and purified by streaking method.

Screening of pesticide-degrading bacteria: The isolates were tested for their ability to degrade selected organophosphates (20 µg mL⁻¹ of diazinon, ethion, chlorpyrifos and profenofos) using eosin-methylene blue agar (EMBA) media indicator (Loos, 1975; Bhat *et al.*, 1994). Isolates were streaked on the surface of each ½ strength EMBA indicator medium containing each different pesticide and incubated for 48 h.

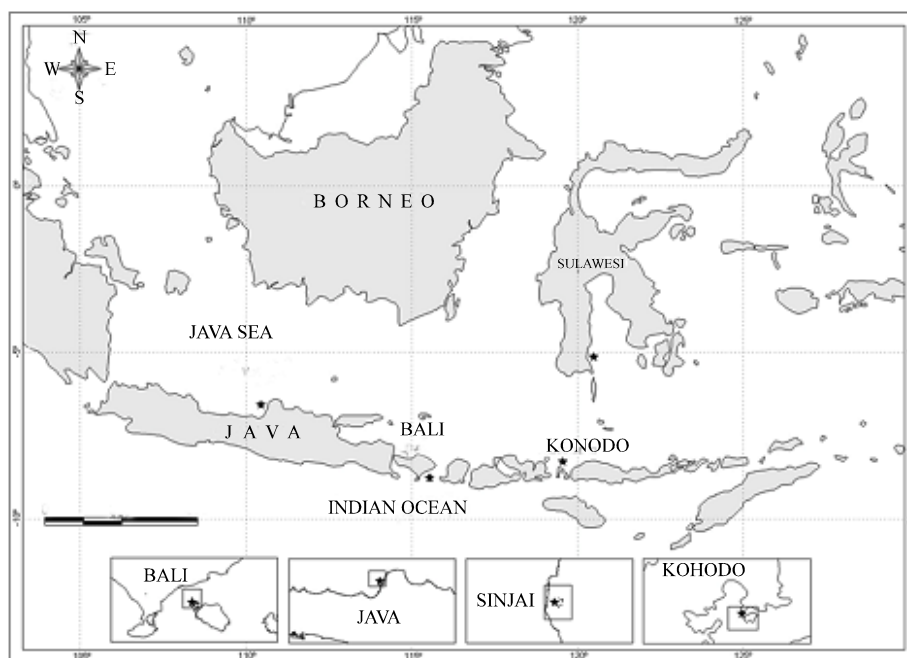


Fig. 1: Coral sampling sites

The color change of colonies into red as an indication of degradative ability was observed. For quantitative determinations of selected organophosphate, isolates that exhibited ability to degrade pesticide in EMBA media indicator were tested by inoculating 0.1 mL amount of an 18 h culture grown in 10 mL of marine broth into 10 mL of sterile seawater with 0.2% peptone containing 20 µg mL⁻¹ of each different type organophosphate and incubated for 5 days at room temperature (28°C) on a rotary shaker. Samples of culture (4×1.5 mL) were removed and centrifuged in a microcentrifuge (Microfuge 11; Beckman Instruments, Inc., Fullerton, Calif) at 12,000 rpm for 3 min and supernatant was decanted into eppendorf. Samples were analyzed immediately for residual organophosphate by GC-MS. The ability of each culture to degrade pesticide was compared with that of a sterile uninoculated control.

DNA extraction and rep-PCR amplification: DNA extraction, PCR amplification of partial 16S rDNA of bacterial strains, purification of PCR products and subsequent sequencing analysis were performed according to the method of Radjasa *et al.* (2007). Primers [(forward primer 8-27: 5'-AGAGTTTGATCCTGGCTCAG-3' (Weisburg *et al.*, 1991) and reverse primer 1510-1492: 5'-GGTTACCTGTTACGACTT-3' (Reysenbach *et al.*, 1992)] were used to amplify 16S rDNA. For Rep-PCR, BOX AIR (5'-CTACGGCAAGGCGACGCTGACG-3') (Versalovic *et al.*, 1994) was used. The REP IR-I and REP 2-1 primers contain the nucleotide inosine (I) at ambiguous positions in the REP consensus. PCR reaction contained of 1 µL DNA template (diluted 100x), 1 µL primer, 7.5 µL Megamix Royal and sterile water up to total volume of 15, 1 µL. Amplifications were performed with a thermal cycler model Gene Amp PCR System 9700 with the following temperature profiles: initial denaturation at 95°C for 5 min; 30 cycles of denaturation (92°C for 1 min), annealing (50°C for 1.5 min), extension (68°C for 8 min) and final extension at 68°C for 10 min. Five microliter aliquot PCR products were run using electrophoresis on 6% acrilamide gel by using 1x TBE buffer.

Grouping of selected isolates was carried out by making matrixes from the positions of bands on the gel which were then analyzed by using Free Tree program by using UPGMA method for constructing the tree. Resampling was performed by bootstrapping with 1000 replications.

Sequencing and phylogenetic analysis: The PCR product was purified and concentrated with Microcon-100 microconcentrators (Amicon, Beverly, MA, USA)

according to manufacturer's instructions. Sequencing was carried out with a Sequi Therm Long-Read Cycle Sequencing Kit (Epicentre Technologies, Madison, WI, USA) and an automated sequencer (the ALF DNA sequences: Pharmacia LKB Biotech, Uppsala, Sweden). A representative of each group was used for sequencing. The resulting 16S rDNA sequences corresponding to the genotype were analyzed for homologies with sequences in the data base using BLAST searching. A phylogenetic tree was constructed using maximum-likelihood analysis. Alignment positions at which less than 50% of sequences of the entire set of data had the same residues were excluded from the calculations to prevent uncertain alignments within highly variable positions of the 16S rDNA. CLUSTAL X was used for multiple alignment/pairwise the DNA sequence (Thompson *et al.*, 1997).

Nucleotide sequence accession numbers: The sequences obtained in this study have been assigned in the GenBank database under accession No. AB449753 to AB4497.

RESULTS AND DISCUSSION

Four different coral life-forms massive (*Porites* sp.), sub-massive (*Galaxea* sp.), branching (*Acropora* sp.) and foliose (*Pavona* sp.) were sampled from four different sampling sites (Java, Bali, Sulawesi and Komodo island) as the source of coral-associated bacteria. Based on the screening by using EMBA indicator medium and degradation test, 25 coral-associated bacteria capable of degrading organophosphorous pesticides were selected. The detail description of organophosphorous pesticide-degrading bacteria is shown in the Fig. 2 and

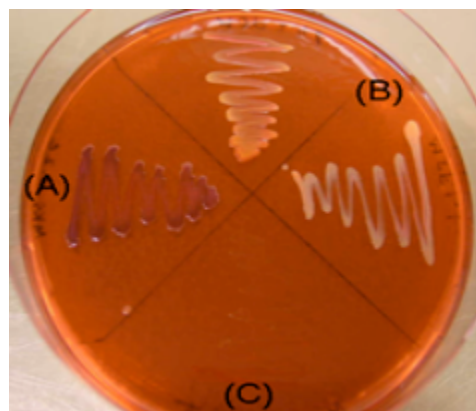


Fig. 2: Screening of pesticide-degrading coral bacteria (A) degraded, (B) no degraded and (C) no growth

Table 1: Microbial degradation of organophosphates as a sole carbon source
Organophosphates degraded (%)

Isolates	Chlorpyrifos	Profenofos	Ethion	Diazinon
KM5	22.44	34.40	-	17.75
KM8	27.50	2.54	-	12.15
KF4	44.40	2.11	-	55.67
KB1	-	32.20	-	-
JM3	53.35	56.75	22.24	-
JM22	20.00	21.54	2.21	2.54
JM27	35.55	12.35	-	-
JM32	54.55	22.12	-	-
JM33	12.22	62.12	2.56	4.56
JS11	2.12	-	4.50	40.00
JS12	1.55	-	7.53	12.15
JB10	33.77	-	22.30	10.10
JB11	12.23	-	11.87	-
JB15	27.70	32.98	24.45	-
SS5	35.12	2.32	-	15.75
SS11	1.75	4.45	-	20.00
SS12	2.25	-	2.50	-
SF14	35.75	-	-	-
SB3	43.30	23.40	53.22	-
BM1	50.45	21.22	1	-
BM5	7.50	40.40	-	-
BS5	20.20	-	33.50	12.60
BF3	-	-	-	25.55
BY6	55.50	-	-	17.25
BF5	3.50	44.24	5.50	5.67

: No growth

Table 1. The degradation pattern as previously indicated by using EMBA indicator medium, multiple (chlorpyrifos, profenofos, ethion and diazinon) degradation property of some isolates was also found in broth medium, however only 24% of the isolates tested showed 50% or greater utilization of organophosphorous as the sole carbon source.

Based on the repetitive-PCR results and constructed dendrogram of the isolates, were created at which similarity level (Fig. 3). The 16S rDNA from strain KM5, JM33, BM5, SB3, KF4 and BY6 representing each of the six groups, were sequenced to obtain information on their identity. BLAST analysis of representative bacterial strains is shown in Table 2. Alignment with the sequences available in the GenBank database, revealed the presence of three major groups of bacteria: (i) *Actinobacteria*, (ii) λ -proteobacteria and (iii) *Bacillus*. The bacterial strains KM5, JM33 and BM5 were found to be members of the *Actinobacteria*. BLAST analysis of bacterial strain KM5 revealed that this strain is a close relative, with 97% similarity, of *Brachybacterium* sp. K473, which has been isolated from deep-water marine invertebrates

Table 2: Characterization of representative pesticide-degrading coral bacteria

Strain	GeneBank Accession No.	Group	Closest relative	Homology (%)	No. of isolate
KM5	AB449755	Actinobacteria	<i>Brachybacterium</i> sp. K473.	97	4
JM33	AB449756	Actinobacteria	<i>Kytococcus sedentarius</i> 13	98	4
BM5	AB449757	Actinobacteria	<i>Brevibacterium</i> sp. HPC407	90	4
SB3	AB449758	α -Proteobacteria	<i>Chromohalobacter salexigens</i> JL816	99	6
KF4	AB449754	Bacillus	<i>Oceanobacillus oncorhynchi</i> R-2	97	4
BY6	AB449753	Bacillus	<i>Bacillus</i> sp. SP8	100	3

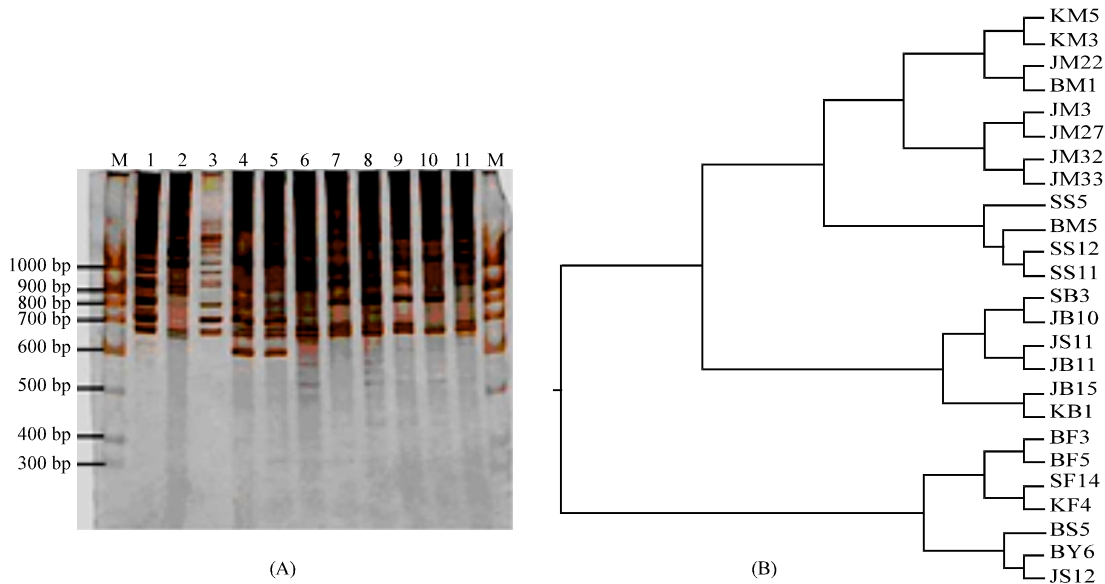


Fig. 3: (A) Part of rep-PCR fingerprint patterns of genomic DNA (B) dendrogram of organophosphate-degrading coral bacteria. (M: DNA markers; 1-11 and KM4-JS12: bacterial isolates)

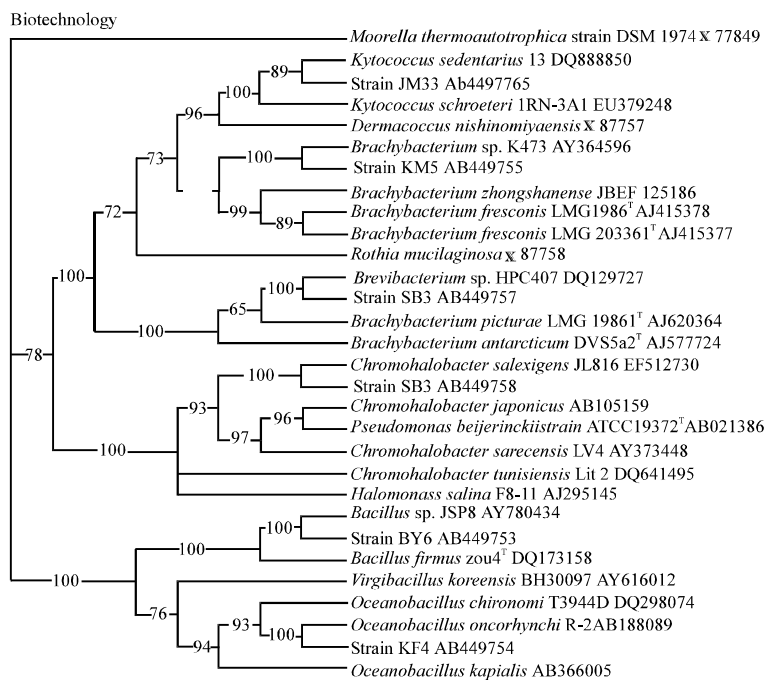


Fig. 4: Phylogenetic tree based on the 16S ribosomal DNA sequence data showing the relationships of representative strains with the most closely related bacteria identified in the GenBank database. *M. thermoautotrophica* was used as out group. Bar indicated 2% dissimilarity of sequences.

(marine cnidarian). The two strains, JM33 and BM5, were closely related to *Kytococcus sedentarius* 13 and *Brevibacterium* sp. HPC407, isolated from sponge *Agelas oroides* in the Mediterranean Sea and activated biomass of an effluent treatment plant (ETP) treating wastewater from pesticide industries, respectively. The bacterial strain SB3 was found to be members of the γ -*Proteobacteria* by 16S rRNA gene sequence analysis. BLAST analysis of bacterial strain SB3 revealed that this strain is a close relative, with 99% similarity, of the strain *Chromohalobacter salexigens* JL816, which has been isolated from marine environments. Strain KF4 and BY6 were found to be members of the *Bacillus* by 16S rRNA gene sequence analysis. BLAST analysis of bacterial strain KF4 revealed that this strain is a close relative, with 97% similarity, of the strain *Oceanobacillus oncorhynchi* R-2, which has been isolated from from the skin of a rainbow trout (*Oncorhynchus mykiss*). Whereas, the strain BY6 is close relative of the strain *Bacillus* sp. SP8 (100%), which has been isolated from Caribbean sponge. To estimate genetic affiliation of the pesticide-degrading isolates among coral-associated bacteria, a neighbor-joining tree including identified isolates and representative marine microorganisms is constructed. A phylogenetic analysis of the 16S rRNA data for selected

strains belonging to the group of the *Proteobacteria*, *Actinobacteria* and *Bacillus* produced the dendrogram shown in Fig. 4. This comparison was made to determine the species to which the six selected isolates are most closely related and to determine how closely the six taxa are related to each other. The phylogenetic analysis indicated that the strain KM5, JM33, BM5, SB3, KF4 and BY6 were closely related to *Brachybacterium* sp., *Kytococcus* sp., *Brevibacterium* sp. *Chromohalobacter* sp., *Oceanobacillus* sp. and *Bacillus* sp., respectively. The sequence results indicate that a high diversity of bacterial phylotypes was present within the corals. The current approach to bioremediation of contaminated waste sites relies primarily on the use of strains indigenous to the site, in these cases treatment consists of modifying environmental variables to enhance microbial growth (Greer *et al.*, 1992; Esposito *et al.*, 1998; Guerin, 1999). Considerable progress has been made in the isolation and development of bacterial strains, which degrade pesticide pollutants. However, most studies have been concerned with soil species (Bending *et al.*, 2003; Racke *et al.*, 1996; Singh *et al.*, 2002; Cullington and Walker, 1999; Guerin, 1999) and less attention has been paid to marine bacterial strains with pesticide degradative activities.

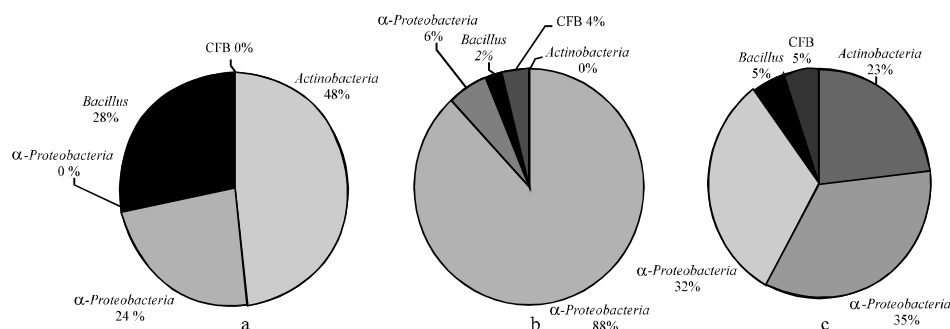


Fig. 5: (a) Differences between coral-associated bacterial communities of isolates from the surface of the four life-form Indonesian corals (this study), (b) isolates from the whole tissue of the Caribbean *M. franksi* (Rohwer *et al.*, 2001) and (c) isolates from the mucus of the Red Sea coral *F. scutaria* (Lampert *et al.*, 2006). CFB, Cytophaga-Flavobacter/Flexibacter-Bacteroides

Coral surfaces are mostly covered by mucopolysaccharide providing a matrix for bacterial colonization for the establishment biofilm-forming bacterial community. Thus, we began with studies on coral-associated bacteria to determine not only their diversity which up to now has been ignored despite their abundance in coral reef communities (Kushmaro *et al.*, 1997; Rohwer *et al.*, 2002), but also their possible ecological role in their natural environments for removal of pesticide pollutants. In the present study, a total of 25 strains were screened for their degradative pattern of four different types of pesticide, namely diazinon, chlorpyrifos, profenofos and ethion.

The primers yielded multiple DNA products ranging in wide size. A visual examination of banding patterns indicated that the coral bacteria were genetically diverse. However, some of the isolates were closely related to each other. Other strains had various degrees of relatedness to each other on the basis of the number of shared PCR products. The culturable organophosphorous pesticides-degrading bacterial community of the tissues of the four life-forms Indonesian stony corals is composed of the bacterial groups *Bacillus*, γ -Proteobacteria and of *Actinobacteria*. When we compared the composition of bacterial isolates of the coral *M. franksi* (Rohwer *et al.*, 2001) and *F. scutaria* (Lampert *et al.*, 2006) to the present study (Fig. 5), we found that, while in *M. franksi* the isolates are dominated mainly by γ -proteobacteria and in *F. scutaria* there is almost an equal distribution of the bacterial groups α -proteobacteria, γ -proteobacteria and of *Actinobacteria*. In this present study, the majority isolates are dominated mainly by *Actinobacteria*. It is not surprising that *Actinobacteria* was found in the present study, since the member of this bacterial group was also found in the previous study (Lampert *et al.*, 2006).

The actinomycetes are prolific producers of novel biologically active natural products. Many of these marine metabolites show great promise as new pharmaceutical agents to treat human ailments (Mincer *et al.*, 2002; Jensen *et al.*, 2005). In nature, actinomycetes are involved in the degradation of lignocellulose (Zimmermann and Broda, 1989), polyester (Tseng *et al.*, 2007), polyaromatic hydrocarbons (Pizzul *et al.*, 2006) and p-Nitrophenol (Hanne *et al.*, 1993). Several studies have also shown their ability to degrade chlorinated aromatic compounds and have elucidated the degradation pathways of some model compounds (Zimmermann and Broda, 1989; Winter *et al.*, 1991). Different members of bacterial groups have been reported to degrade organophosphorous compounds (Horne *et al.*, 2002; Zhongli *et al.*, 2001; French *et al.*, 1998; Karpouzias and Walker, 2000). However, this is the first report of organophosphate degradation by class of *Actinobacteria* isolated from corals. Actinomycetes could therefore be candidates for the biological treatment of marine pollutants.

Present analyses showed that the bacterial diversity associated with corals in the present study is smaller than that of the previous reported. The culturable bacterial communities in this study did not find any members of γ -proteobacteria and CFB bacterial group. The medium used is probably the reason why a different composition of heterotrophic bacteria was detected. These earlier studies used glycerol artificial seawater medium (GASW) and Bacto Marine Agar 2216 that are nonselective media. Whereas, we used selective medium by adding organophosphorous pesticide compounds as an isolation medium. In addition, the material source of bacterial strains found in this study were isolated from the coral surface, as opposed to the coral mucus layer (Lampert *et al.*, 2006) and the coral whole tissue

(Rohwer *et al.*, 2001) used in previous studies. It is clear that the currently known coral-associated microbial diversity is strongly affected by the cultivation conditions. However, the present study revealed the noteworthy of isolation and studying the genetic diversity of coral-associated bacteria and at least in part, clarified the population structure of organophosphorous pesticide-degrading bacteria in coral reefs ecosystem of the Indonesia coastal waters.

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